

# Redefining the In Vivo Origin of Metanephric Nephron Progenitors Enables Generation of Complex Kidney Structures from Pluripotent Stem Cells

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## SUMMARY

Recapitulating three-dimensional (3D) structures of complex organs, such as the kidney, from pluripotent stem cells (PSCs) is a major challenge. Here, we define the developmental origins of the metanephric mesenchyme (MM), which generates most kidney components. Unexpectedly, we find that posteriorly located T<sup>+</sup> MM precursors are developmentally distinct from Osr1<sup>+</sup> ureteric bud progenitors during the postgastrulation stage, and we identify phasic Wnt stimulation and stage-specific growth factor addition as molecular cues that promote their development into the MM. We then use this information to derive MM from PSCs. These progenitors reconstitute the 3D structures of the kidney in vitro, including glomeruli with podocytes and renal tubules with proximal and distal regions and clear lumina. Furthermore, the glomeruli are efficiently vascularized upon transplantation. Thus, by reevaluating the developmental origins of metanephric progenitors, we have provided key insights into kidney specification in vivo and taken important steps toward kidney organogenesis in vitro.

## INTRODUCTION

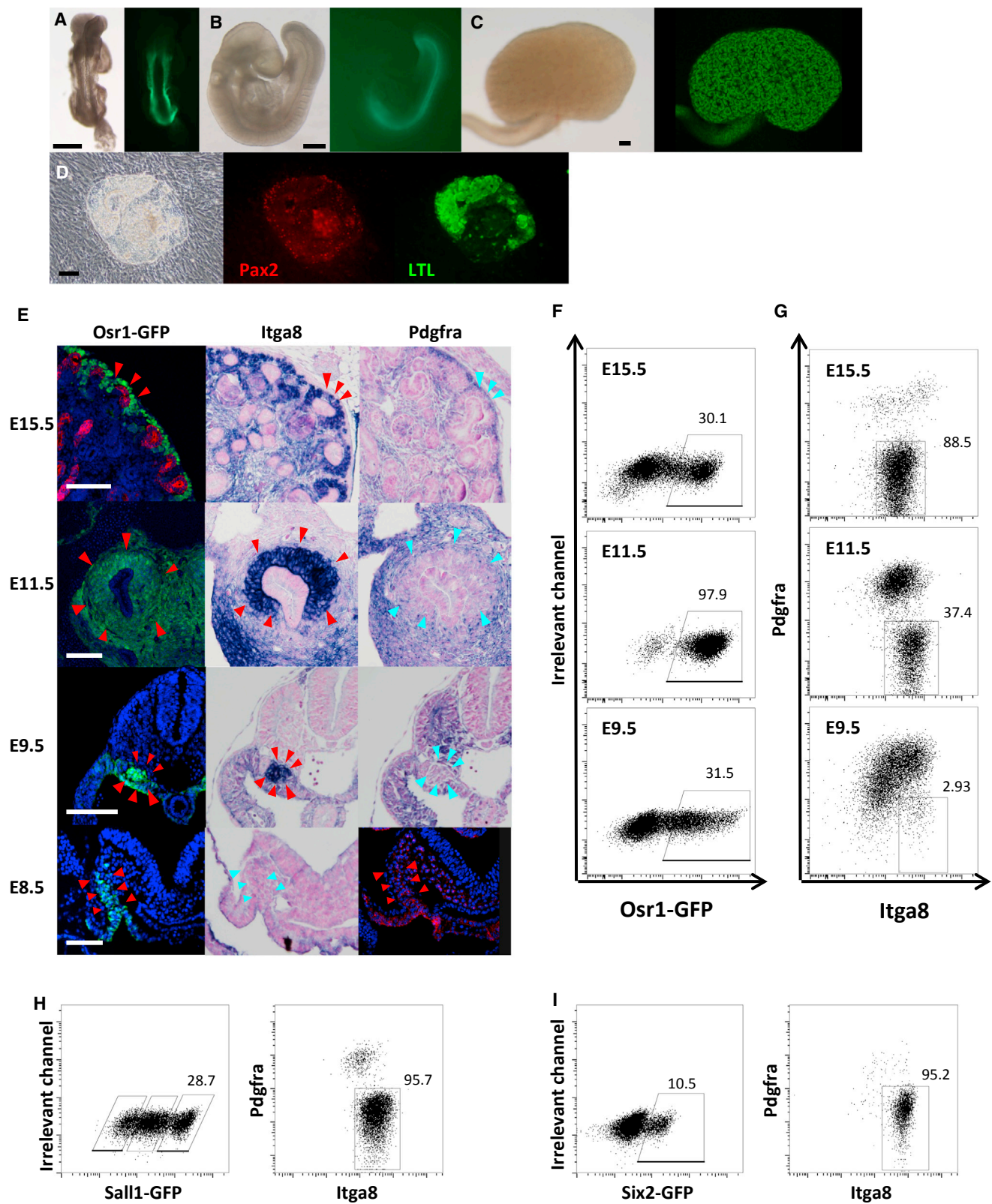
Despite the successful achievement of various types of tissues from pluripotent stem cells (PSCs), kidney generation in vitro has remained a challenge for developmental biology and regenerative medicine (Murry and Keller, 2008; Williams et al., 2012). This is partly because the specification of the kidney lineage in vivo has not been well clarified, owing to its complex processes involving the formation of three consecutive primordia (pronephros, mesonephros, and metanephros) during embryogenesis (Saxen, 1987). The pronephros and mesonephros eventually degenerate in females, whereas a portion of the

mesonephros in males contributes to a drainage system for the future testis, including the epididymis. The kidney derives from the embryonic metanephros, which develops at the most posterior part of the body trunk. The metanephros is formed by reciprocally inductive interactions between two precursor tissues, namely the metanephric mesenchyme (MM) and the ureteric bud. Cell fate analyses have shown that both the MM and ureteric bud derive from the intermediate mesoderm, which appears around embryonic day 8.5 (E8.5) and expresses the transcription factor *Osr1* (Mugford et al., 2008b). However, the mechanisms underlying how the nascent mesoderm becomes committed to the intermediate mesoderm and how the MM and ureteric bud lineage segregate from one another have not been clarified, despite many reports showing the importance of various growth factors during kidney development (Costantini and Kopan, 2010; Fleming et al., 2013; Kim and Dressler, 2005; Moriya et al., 1993; Poladia et al., 2006). Moreover, it remains to be elucidated how the anteroposterior axis is formed along the intermediate mesoderm and gives rise to the posteriorly located metanephros. In the present study, we addressed these questions by in vivo lineage-tracing experiments together with in-vitro-directed differentiation systems utilizing sorted embryonic kidney precursors at each developmental stage. Importantly, by establishing conditions for the later stages first, and then moving backward to the earlier stages, we were able to optimize the multistep culture conditions from embryonic precursors toward nephron progenitors. Finally, the protocol established by these strategies was successfully applied to the induction of metanephric nephron progenitors from mouse embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs), thereby advocating a model for kidney lineage specification.

## RESULTS

### The Osr1<sup>+</sup>/Integrin8<sup>+</sup>/Pdgfra<sup>+</sup> Population Represents Colony-Forming Nephron Progenitors

The MM gives rise to the epithelia of glomeruli (i.e., podocytes) and renal tubules, which constitute the major parts of the nephrons, as shown by cell fate analyses involving labeling of



**Figure 1. Nephron Progenitors Exist in the  $Osr1^{+}/Itga8^{+}/Pdgfra^{-}$  Population**

(A–C) EGFP expressions in  $Osr1$ -GFP knockin mice at E8.5 (A), E9.5 (B), and E15.5 (C) are shown. Scale bars, 500  $\mu$ m.

(D) Bright-field image and immunostaining of a colony are presented. Scale bar, 100  $\mu$ m.

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mesenchyme expressing the transcription factor *Six2* (Kobayashi et al., 2008). We previously proved the presence of nephron progenitors by establishing a colony-formation assay. When we plated dissociated metanephric mesenchymal cells, which strongly express *Sal1*, onto feeder cells stably expressing *Wnt4*, single cells formed colonies that expressed glomerular and renal tubule markers (Nishinakamura et al., 2001; Osafune et al., 2006). Therefore, the *Sal1*<sup>high</sup> and *Six2*<sup>+</sup> MM represents a nephron progenitor population in the embryonic kidney.

*Osr1* is another MM marker and also one of the earliest markers for the intermediate mesoderm and is thus continuously expressed in the renal precursor population throughout kidney development (James et al., 2006; Mugford et al., 2008b). We generated *Osr1*-GFP knockin mice (Figures S1A–S1C available online) and confirmed that GFP was expressed in the intermediate mesoderm at E8.5–E9.5 and the MM at E11.5–E15.5 (Figures 1A–1C). Similar to our previous experiments, the *Osr1*-GFP<sup>+</sup> population sorted from E11.5 and E15.5 embryonic kidneys contained colony-forming nephron progenitors (Table S1). The colonies expressed nephric transcription factors, such as *Pax2* and *Sal1*, as well as markers for differentiated renal tubules (Figures 1D and S1D). Therefore, the *Osr1*<sup>+</sup> MM contains colony-forming nephron progenitors.

Next, we searched for cell surface markers that can further enrich the nephron progenitors. At E15.5 and E11.5, *Itga8* (*Itga8*) was strongly expressed in the capping mesenchyme around the ureteric bud tips, whereas *Pdgfra* was excluded from the population (Li et al., 2000; Müller et al., 1997) (Figure 1E). By fluorescence-activated cell sorting (FACS) analyses, we identified the existence of *Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> fractions in the *Osr1*-GFP<sup>+</sup> populations of E15.5 and E11.5 embryonic kidneys (Figures 1F and 1G), and colony-forming nephron progenitors were enriched in the *Osr1*<sup>+</sup>/*Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> fractions (Table S2). It is of note that not all cells in the *Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> fraction of each specimen were positive for *Osr1*-GFP, meaning that all three markers were required for identification of the nephron progenitor populations (Figure S1E). We further confirmed the reliability of these cell surface markers using GFP knockin mice for *Sal1* and *Six2*, in which most of the *Sal1*-GFP<sup>high</sup> or *Six2*-GFP<sup>+</sup> cells were *Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> (Figures 1H and 1I). Therefore, the *Osr1*-GFP<sup>+</sup>/*Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> fraction represents the colony-forming nephron progenitors.

### The Anterior Intermediate Mesoderm at E9.5 Contains Colony-Forming Progenitors that Contribute to the Mesonephros

We next examined the expressions of nephron progenitor markers and the colony-forming abilities of *Osr1*-GFP<sup>+</sup> cells at earlier stages. At E8.5, we did not detect any overlap of GFP with *Itga8*, and no colonies were formed by the GFP<sup>+</sup> population (Figure 1E; Table S1). At E9.5, we detected colony formation by the GFP<sup>+</sup> population (0.037% ± 0.013%; Table S1; Figures S2A

and S2B). We also found a GFP<sup>+</sup> region that was *Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> (Figure 1E), and sorting of the *Osr1*<sup>+</sup>/*Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> population enriched the colony-forming cells (1.10% ± 0.26%; Figures 1F and 1G; Table S2). However, even after the enrichment, the colony-forming frequency was significantly lower than those of the *Osr1*<sup>+</sup>/*Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> populations from the metanephric region at E10.5 and E11.5 (30.9% ± 1.5% and 50.9% ± 5.2%, respectively; Table S2). In contrast, the colony-forming frequency of the *Osr1*<sup>+</sup>/*Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> population from the mesonephric region at E10.5 (1.47% ± 0.20%) was as low as that from E9.5. Because the mesonephros, which is located anterior to the metanephros, develops earlier than the metanephros and forms much fewer nephrons (Saxen, 1987), we hypothesized that the colony-forming cells at E9.5 may represent mesonephric nephron progenitors. Indeed, colony-forming progenitors were only detected among the anterior part of GFP<sup>+</sup> cells in the E9.5 embryo (Figure S2C). Furthermore, *Pax2* and *Six2*, which mark nephron progenitors, were predominantly expressed in the anterior part of the intermediate mesoderm (Figure 2A). We further crossed *Six2*-GFP<sup>CreER</sup> mice (Kobayashi et al., 2008) with mice carrying a *tdTomato* reporter allele (Madisen et al., 2010) and injected tamoxifen to transiently activate Cre in the anterior intermediate mesoderm at E9.5. When analyzed at E11.5, labeled cells were detected in the mesonephros, but not in the metanephros (Figure S2D). Therefore, the anterior and posterior parts of the *Osr1*-GFP<sup>+</sup> intermediate mesoderm at E9.5 have different characteristics, and the anterior intermediate mesoderm, which contributes to the mesonephros, may not give rise to the metanephric nephron progenitors.

### Metanephric Nephron Progenitor Induction from the Posterior Intermediate Mesoderm at E9.5

To further investigate the molecular differences and required factors between the early stages of kidney precursor development, we performed microarray and quantitative RT-PCR analyses using the *Osr1*<sup>+</sup>/*Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> colony-forming presumptive mesonephric progenitors at E9.5 and metanephric nephron progenitors at E10.5–E11.5 (Figures 2B and S3A). Although both types of progenitors expressed many transcription factors in common, such as *Osr1*, *Wt1*, *Pax2*, and *Six2*, the metanephric progenitors expressed posterior *Hox* genes including *Hoxa10*, *Hoxa11*, and *Hoxd12* more abundantly. The *Hox11* family genes, which start to be expressed at the posterior end of the embryo around E9.0, are essential for metanephros development by dictating the metanephric region along the anterior-posterior axis in the intermediate mesoderm (Mugford et al., 2008a; Nelson et al., 2008; Wellik et al., 2002). Furthermore, a cell fate-mapping study showed that the *Osr1*<sup>+</sup> intermediate mesoderm at E9.5 contributes to the MM (Mugford et al., 2008b). Therefore, we hypothesized that the non-colony-forming *Osr1*<sup>+</sup>/*Wt1*<sup>+</sup>/*Pax2*<sup>−</sup>/*Six2*<sup>−</sup>/posterior

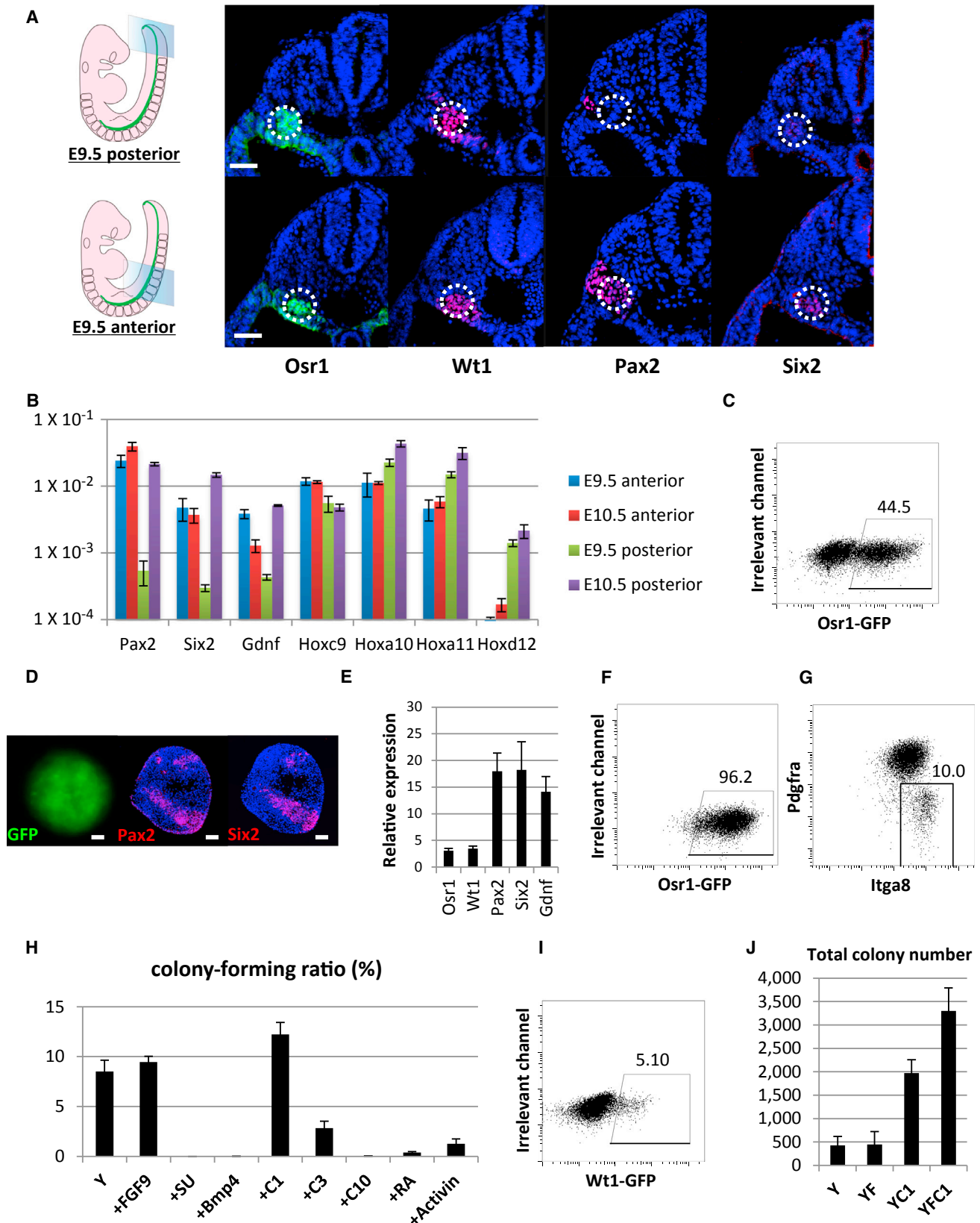
(E) Immunostaining of embryonic sections is shown. Red color staining shows the CK8 expression in the ureteric bud (E15.5). The arrowheads indicate the capping mesenchyme at E15.5 and E11.5, condensed intermediate mesoderm at E9.5, and intermediate mesoderm at E8.5. Scale bars, 100 μm.

(F and G) FACS analyses of *Osr1*-GFP (F) and *Itga8*/*Pdgfra* (G) in the embryos at each indicated stage are shown.

(H and I) FACS analyses of the *Sal1*-GFP<sup>high</sup> population (H) and *Six2*-GFP<sup>+</sup> population (I) in the E11.5 metanephros and of *Itga8*/*Pdgfra* expressions are presented.

See also Figures S1 and S2, and Tables S1 and S2.





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*Hox*<sup>+</sup> intermediate mesoderm, which was located posteriorly at E9.5, could be a precursor population for the metanephric nephron progenitors.

To test our hypothesis, we sorted *Osr1*-GFP<sup>+</sup> cells from the posterior part of E9.5 embryos and plated them into low-cell-binding plates in the presence of the Rho kinase inhibitor Y27632, which supports cell survival (Watanabe et al., 2007) (Figures 2C and S3B). The cells spontaneously reaggregated and formed spheres within 24 hr. At 48 hr of culture, the aggregates retained intense GFP signals and showed more than 10-fold higher expressions of *Pax2*, *Six2*, and *Gdnf* compared with the starting point (Figures 2D–2F). FACS analyses also showed the emergence of an *Osr1*<sup>+</sup>/*Itga8*<sup>+</sup>/*Pdgfra*<sup>−</sup> population (10.0% ± 0.01% of the total cells; Figures S3C and 2G). When these aggregates were dissociated and stimulated with Wnt4, colony formation was observed (Figure 2H), suggesting in vitro derivation of metanephric nephron progenitors from the posterior intermediate mesoderm.

We further addressed the effects of growth factors in this process. The microarray data described above showed the accumulated expressions of Fgf ligands (especially *Fgf9* and *Fgf20*), receptors, and their downstream target genes in the colony-forming populations at both E9.5 and E11.5, whereas the Bmp and Wnt targets were downregulated (Figure S4A). Indeed, inhibition of Fgf signaling, exogenous addition of Bmp4, or high concentrations of a Wnt agonist (3 or 10 μM CHIR99021 [CHIR]), as well as addition of retinoic acid or activin, were inhibitory for the induction of colony-forming progenitors and nephric gene expressions (Figures 2H and S4B), whereas addition of FGF9 or a low concentration of the Wnt agonist (1 μM CHIR) slightly improved the colony formation (Figure 2H). Because *Osr1*-GFP is expressed by a rather broad population, including the lateral plate mesoderm, we examined the efficacy of these factors using *Wt1*-GFP knockin mice (Zhou et al., 2008), which show more restricted expression in a region of the intermediate mesoderm (Figures 2A, 2C, and 2I). Addition of Fgf9 and a low dose of the Wnt agonist synergistically promoted sphere growth and increased colony formation by the spheres (Figures 2J and S4C).

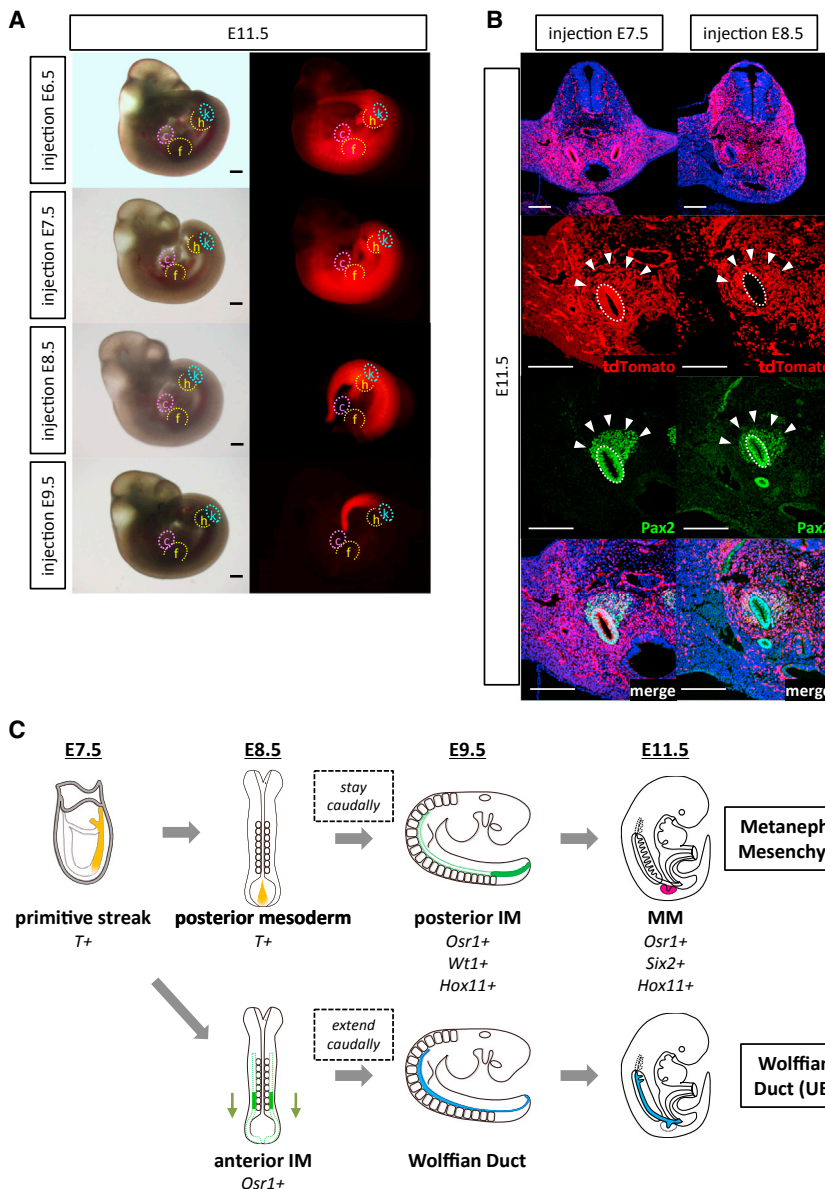
Thus, the combination of 1 μM CHIR and Fgf9 (C1F) was optimal for induction of metanephric nephron progenitors from the posterior intermediate mesoderm. These observations are consistent with previous findings showing requirements of Fgf receptors and Fgf9/Fgf20 for the formation and maintenance of the MM, respectively (Barak et al., 2012; Poladia et al., 2006).

### The Precursor of the MM Is Maintained in the T<sup>+</sup> Caudal Population until the E8.5 Postgastrulation Stage

Next, we searched for an in vitro method to differentiate the mesoderm at earlier stages into MM by way of the E9.5 posterior intermediate mesoderm. One report has shown that both the MM and ureteric bud derive from the intermediate mesoderm, which appears around E8.5 and expresses *Osr1* (Mugford et al., 2008b). Several other reports have shown that the ureteric bud originates from the anterior intermediate mesoderm, and its anlage, the Wolffian duct, elongates in an anterior-to-posterior manner, as demonstrated by direct labeling in chick embryos (Atsuta et al., 2013; Attia et al., 2012; Obara-Ishihara et al., 1999; Saxen, 1987). In murine embryos, the *Pax2*<sup>+</sup>/*Pax8*<sup>+</sup> anterior intermediate mesoderm at E8.5, called the pronephric anlage, is supposed to be the equivalent population (Bouchard et al., 2002) and is included in the *Osr1*<sup>+</sup> region. Thus, we initially examined the effects of many combinations of growth factors on sorted E8.5 *Osr1*-GFP<sup>+</sup> cells. However, we were unable to induce colony-forming progenitors. Unexpectedly, we experienced colony formation from the *Osr1*-GFP<sup>−</sup> fraction when treated with growth factors. Therefore, we hypothesized that the posterior immature mesoderm, which was negative for *Osr1*, contained prospective nephron progenitors. Deletion of *T* (*Brachyury*), a representative marker of the primitive streak and posterior nascent mesoderm, causes a caudal truncation that includes the metanephric region (Herrmann et al., 1990). We first crossed *T<sup>trEGFP-CreERT2</sup>/+* mice (Imuta et al., 2013) with mice carrying the *tdTomato* reporter allele and injected tamoxifen at the gastrulation stage (E6.5 and E7.5), when the initial germ layer formation takes place (Murry and Keller, 2008; Tam and Behringer, 1997). When analyzed at E11.5, most of the mesodermal tissues, including the heart, limbs, and kidneys, were labeled (Figures 3A and 3B). Next, we injected tamoxifen at E8.5 and found that labeled cells were only detected in the lower trunk of the E11.5 embryo, including the MM located at the hindlimb level (Figure 3A). On the other hand, the heart and forelimbs, which are anteriorly located mesodermal tissues, were no longer labeled (Figure 3A). Surprisingly, when we created sections at the metanephric level, only the MM, and not the ureteric bud, was labeled (Figure 3B). These findings indicate that at E8.5, the precursor of the ureteric bud was located anteriorly in the T<sup>−</sup> population and already segregated from that of the MM, which was localized in T<sup>+</sup> posterior nascent mesoderm. These data are consistent with the notion that the ureteric bud originates from the anterior intermediate mesoderm at E8.5 and

### Figure 2. Metanephric Nephron Progenitor Induction from the E9.5 Posterior Intermediate Mesoderm

- (A) Immunostaining for the indicated regions is presented. The intermediate mesoderm is outlined by the dashed lines. Scale bars, 50 μm.  
 (B) E9.5 and E10.5 anterior are the mesonephric progenitors, E10.5 posterior is the metanephric progenitors, and E9.5 posterior is the caudal intermediate mesoderm. The relative expression of each transcript to β-actin expression is presented as the mean ± SEM (n = 3).  
 (C) *Osr1*-GFP<sup>+</sup> population gated for cell sorting is shown.  
 (D) GFP and immunostaining of cultured cell aggregates are shown. Scale bars, 100 μm.  
 (E) Relative expression of each transcript to the starting material is presented as the mean ± SEM (n = 3).  
 (F and G) FACS analyses of *Osr1*-GFP expression (F) and *Itga8*/*Pdgfra* expression (G) are shown.  
 (H) Colony-forming ratios of cultured cell aggregates are presented as the mean ± SEM (n = 3). Y, Y27632 (Rock inhibitor); SU, SU5402 (Fgf1 inhibitor); C1, 1 μM CHIR (canonical Wnt agonist); C3, 3 μM CHIR; C10, 10 μM CHIR; RA, retinoic acid.  
 (I) *Wt1*-GFP<sup>+</sup> population gated for cell sorting is shown.  
 (J) Total colony numbers from cultured cell aggregates are presented as the mean ± SEM (n = 5). F, Fgf9.  
 See also Figures S2–S4.



**Figure 3. The MM Is Derived from the Posterior Mesoderm Maintained in the  $T^+$  Nascent State until E8.5**

(A) Lateral view shows E11.5 whole embryos (left panels) and comparisons of fluorescent labeling after injection of tamoxifen at various stages. The regions of the heart (c), metanephric kidney (k), forelimb (f), and hindlimb (h) are indicated by dashed lines. Scale bars, 500  $\mu$ m.

(B) Immunostaining of an E11.5 embryo labeled by injections of tamoxifen at E7.5 and E8.5 is shown. The arrowheads indicate the MM, and the ureteric buds are outlined by dashed lines. Scale bars, 100  $\mu$ m.

(C) Model for lineage segregation of the ureteric bud (UB) and the MM is shown. IM, intermediate mesoderm.

See also Figure S5.

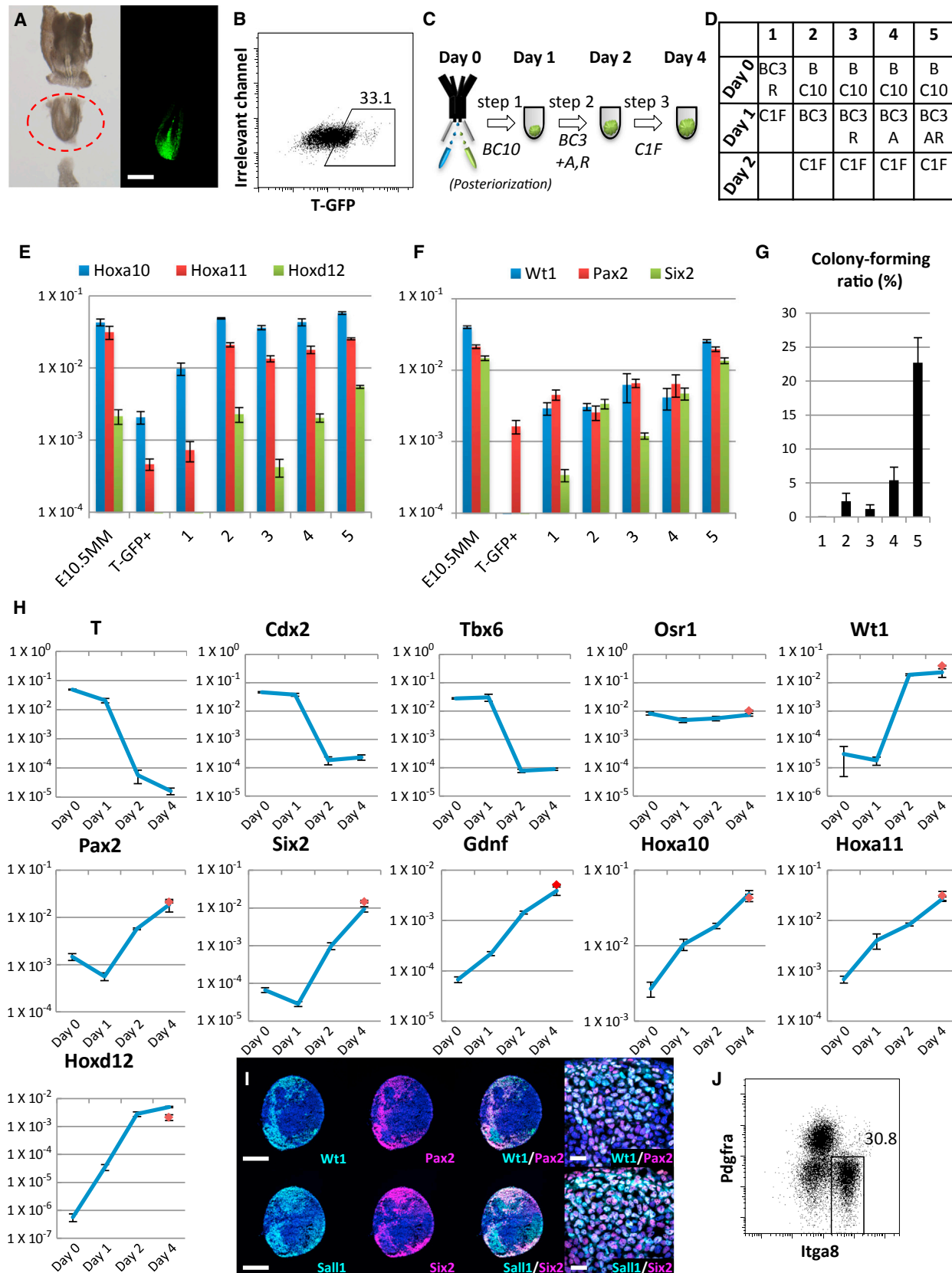
### MM Induction from the $T^+$ Caudal Precursor at E8.5

Based on the lineage-tracing experiments, we used the sorted E8.5  $T^+$  posterior mesoderm as the starting material to induce metanephric nephron progenitors (Figures 4A–4C). The sorted cells were re-aggregated to form spheres, followed by treatment with C1F as described above (Figure 4C, step 3). Because the posterior intermediate mesoderm in E9.5 embryo was marked by expressions of *Osr1*, *Wt1*, and posterior *Hox* genes, we first focused on the growth factors that may affect the expressions of these genes. Synergistic effects of Bmp and Wnt signaling have been reported on the expressions of posterior *Hox* genes in mouse ESC differentiation (Lengerke et al., 2008), and retinoic acid signaling is important for the expressions of *Wt1* homologs in zebrafish development (Bollig et al., 2009). Because simultaneous

elongates caudally (Figure 3C). In contrast, when we injected tamoxifen at E9.5, only the tail region was labeled, and no labeled cells were observed in the MM region (Figure 3A), suggesting that the  $T^+$  cells at this stage no longer possess the capacity to differentiate into the MM.

Taken together, our observations indicate that the precursor of the MM is maintained and posteriorized in the  $T^+$  state until the E8.5 postgastrulation stage (Figure 3C). Presumably, this could in part correspond to the “axial progenitor,” which was recently recognized as the source of the caudal body trunk (Wilson et al., 2009). These data also reveal differences in the developmental processes between the posteriorly located MM and the anteriorly located mesodermal tissues, such as the heart, which have been successfully induced from PSCs by way of the  $T^+$  state in the initial short period of differentiation (BurrIDGE et al., 2012).

introduction of retinoic acid, Bmp, and the Wnt agonist did not increase the posterior *Hox* genes to the levels observed in the embryonic MM (Figures 4D and 4E), we added a “posteriorization phase.” Because canonical Wnt signals are reportedly important for caudal body extension (Wilson et al., 2009; Yamaguchi et al., 1999), we added a high concentration of the Wnt agonist (10  $\mu$ M CHIR) in combination with Bmp4. This treatment (Figure 4C, BC10 in step 1) dramatically increased the expressions of posterior *Hox* genes, and we were able to detect colony formation from the induced cells, although the expression levels of nephric genes were still lower than those in the embryonic MM (Figures 4D–4G). We tried various conditions and found that the combination of activin and retinoic acid, together with Bmp4 and 3  $\mu$ M CHIR (Figure 4C, ABC3 in; step 2), substantially increased the expressions of nephric genes (Figure 4F). In this optimized three-step condition (BC10 followed by ABC3R and C1F), the



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cells formed higher numbers of colonies ( $22.7\% \pm 3.66\%$ ,  $n = 4$ ) compared with single addition of either activin or retinoic acid (Figure 4G). In the first posteriorization step, the concentration of the canonical Wnt agonist was critically important, and addition of retinoic acid completely inhibited the colony-forming progenitor induction (Figure S5A). In the second step, inhibition of any one of activin, retinoic acid, Bmp, Wnt, or Fgf signaling resulted in a decrease in colony formation (Figure S5B), suggesting that all of these signals were essential. We further examined the temporal kinetics of the gene expressions at every step of the induction process (Figure 4H). In the first step, *T*, *Cdx2*, and *Tbx6*, all of which are posterior nascent mesoderm markers, were expressed and maintained, whereas posterior *Hox* genes started to increase. In the second step, nephric genes started to be expressed, and in the final step, the expressions of *Pax2*, *Six2*, *Gdnf*, and posterior *Hox* genes increased to the levels in the embryonic MM. We further confirmed the colocalization of multiple transcription factors in single cells, such as *Wt1/Pax2* and *Sall1/Six2* (Figure 4I), and the existence of the *Itga8<sup>+</sup>/Pdgfra<sup>-</sup>* population ( $30.8\% \pm 2.4\%$ ,  $n = 4$ ) in the induced spheres (Figure 4J). Taken together, we established a protocol to induce metanephric nephron progenitors from the posterior nascent mesoderm.

#### Metanephric Nephron Progenitor Induction from Mouse ESCs

Next, we tried to induce metanephric nephron progenitors from ESCs. To monitor the induction of intermediate mesoderm and metanephric nephron progenitors, we employed the *Osr1-GFP* ESC line, from which we generated the *Osr1-GFP* mice described above. Embryoid bodies (EBs) were generated in serum-free medium without any factors for 2 days (Gadue et al., 2006) (Figure 5A). During the following 24 hr, a low concentration of activin induced transient expression of the epiblast marker *Fgf5* (Figure 5B). The EBs were subsequently treated with Bmp4 and a high concentration of Wnt agonist (10  $\mu$ M CHIR). At day 4.5, the expressions of *T*, as well as *Cdx2* and *Tbx6*, which represent the posterior nascent mesoderm, were upregulated. Subsequently, we completely mimicked the protocol for the embryonic posterior mesoderm (Figures 4C and 5A; steps 3–5). The induced EBs harvested at day 8.5 expressed multiple signature genes for metanephric nephron progenitors at comparable levels to the embryonic MM (Figure 5B). Immunostaining showed that many cells coexpressed typical metanephric nephron progenitor markers, including *Osr1*, *Wt1*,

*Pax2*, *Sall1*, and *Six2* (Figure 5C). Furthermore, FACS analyses showed that nearly 90% of the cells were *Osr1-GFP<sup>+</sup>*, and among the *Osr1<sup>+</sup>* cells, *Itga8<sup>+</sup>/Pdgfra<sup>-</sup>* progenitors constituted approximately 65% (Figure 5D). These induced progenitors exhibited robust colony formation ( $21.3\% \pm 1.7\%$ ,  $n = 8$ ). Therefore, we succeeded in generating metanephric nephron progenitors from mouse ESCs. It is of note that the in vivo MM in the E11.5 embryo contains another cell type, stromal cells, located in the outer layer of the nephron progenitors (Humphreys et al., 2010; Mugford et al., 2008b). As shown in Figures 1E and 1G, these stromal cells were included in the *Osr1<sup>+</sup>/Pdgfra<sup>+</sup>/Itga8<sup>low</sup>* population. Although our induced EBs contained some *Pdgfra<sup>+</sup>* cells (Figure 5D), the expressions of stromal cell marker genes, including *Foxd1*, *Rarb*, and *Pbx1* (Hatini et al., 1996; Mendelsohn et al., 1999; Schnabel et al., 2003), were comparable to those in the E11.5 embryonic *Six2<sup>+</sup>* nephron progenitors and much lower than those in the stroma of the kidney (Figure 5E). Therefore, our protocol directs the cells specifically toward the nephron progenitors, but not the stroma.

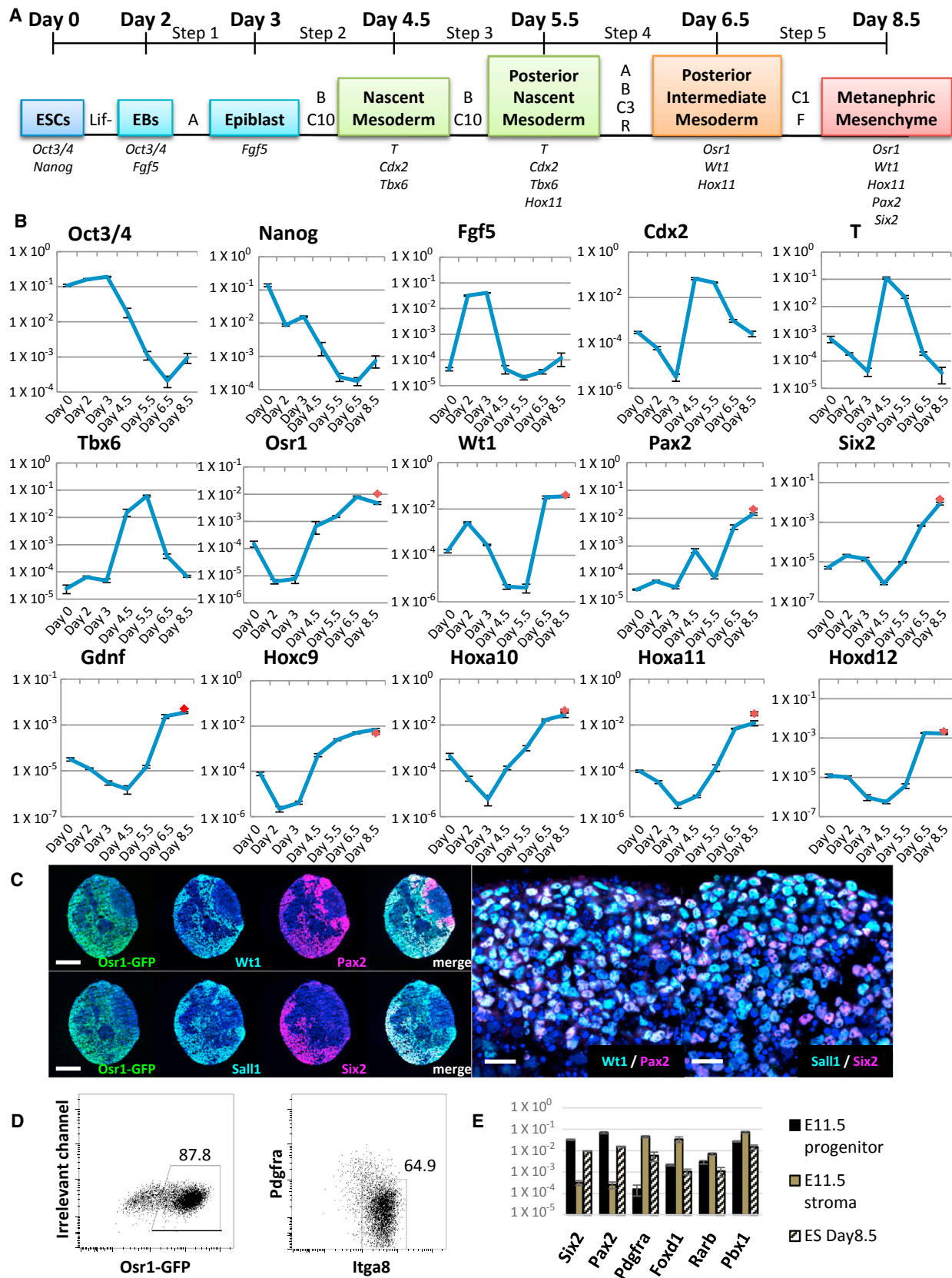
#### 3D Kidney Structures Formed by ESCs

It is well established that the MM from E11.5 embryos undergoes mesenchymal-to-epithelial transition and forms glomeruli and renal tubules when cocultured with embryonic spinal cords or Wnt4-expressing cells at the air-liquid interface (Kispert et al., 1998) (Figures 6A and 6B). Therefore, we cultured the induced EBs in the same manner, which resulted in robust tubulogenesis. Specifically, histological examination of EBs harvested at day 6 identified many tubules under both conditions (Figures 6C, 6D, and S6A). Most of the tubules were positive for *Pax2* and *Sall1*, indicating that they were renal tubules (Figures 6E–6H). Some tubules expressed markers for proximal tubules, such as *LTL*, *Cadherin6*, *Aquaporin1*, *Jagged1*, and *Megalin* (Figures 6F, 6G, and 6L–6N). Other tubules showed expressions of *E-cadherin*, *Brn1*, and *NCC*, indicative of distal tubule formation (Figures 6H, 6O, and 6P). More impressively, numerous glomerulus-like structures were observed (Figures 6D and 6I). These structures contained clusters of cells that expressed a typical podocyte marker, *Wt1*, in their nuclei, as well as foot process proteins such as *nephrin* and *podocin* (Figures 6I–6K). These structures were indistinguishable from those of the embryonic MM (Figures 6A and 6B,  $n = 6$ ). On the other hand, the EBs cultured without factors after step 2 (Figure 5A) showed some neural rosette-like formations, but no kidney tubule or glomeruli formations (Figure S6B).

#### Figure 4. Metanephric Nephron Progenitor Induction from the E8.5 T<sup>+</sup> Posterior Nascent Mesoderm

(A) T-GFP expression in the E8.5 embryo is shown. Bright field (left panel) and the fluorescence (right panel) are presented. Scale bar, 500  $\mu$ m. The presomitic mesoderm region is outlined by the dashed line.  
(B) The gate for T-GFP<sup>+</sup> cell population sorting is shown.  
(C and D) Culture conditions are shown for (E)–(G). A, Activin; B, Bmp4; C, CHIR; R, retinoic acid; F, Fgf9.  
(E and F) Relative expression of each transcript to  $\beta$ -actin expression is presented as the mean  $\pm$  SEM ( $n = 4$ ). E10.5MM, E10.5 Metanephric Mesenchyme; T-GFP<sup>+</sup>, starting sample.  
(G) Colony-forming ratios of cultured cell aggregates are presented. Data are shown as the mean  $\pm$  SEM ( $n = 4$ ).  
(H) Temporal kinetics of marker genes is shown. The expression levels in the E10.5 embryonic MM are shown in red. The relative expression of each transcript to  $\beta$ -actin expression is presented as the mean  $\pm$  SEM ( $n = 4$ ).  
(I) Immunostaining of induced cell aggregates is presented. High-magnification images are shown in the right two panels. Scale bars, 200  $\mu$ m (left six panels) and 20  $\mu$ m (right two panels).  
(J) FACS analysis of *Itga8/Pdgfra* expression at day 4 is shown.  
See also Figures S4 and S5.





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We further used another ESC line with ubiquitous expression of dsRed and transplanted the induced EBs beneath the kidney capsule of immunodeficient mice, together with spinal cords (Figures 6Q, 6R, S6C, and S6D). When harvested after 1 week, the rudiments had undergone massive tubulogenesis, similar to the *in vitro* cultures (Figures 6R–6T). Furthermore, many blood vessels had integrated into the transplanted tissue, including ESC-derived glomeruli (Figures 6U–6W, 22 of 26 glomeruli [84.6%]). Notably, the glomerular podocytes in the graft tissue expressed vascular growth factors, including Vegfa and Ephrin B2, both of which are indispensable for endothelial capillary formation in the glomeruli (Figures 6X and 6Y) (Eremina et al., 2003; Takahashi et al., 2001). Importantly, the vascularized glomeruli contained red blood cells (Figure 6U), and the integrated vessels were negative for dsRed (Figure S6E), indicating that the transplanted glomeruli could become connected to the host circulation, which is an essential requirement for the glomerular function as a filtration apparatus.

### Metanephric Nephron Progenitor Induction from Human iPSCs

We further applied our protocol to human iPSC differentiation toward metanephric nephron progenitors *in vitro*. Previous reports showed the importance of Bmp, Fgf, and activin signals for the initial induction of mesodermal lineage cells for human PSCs (Bernardo et al., 2011; Kattman et al., 2011). Therefore, we treated human iPSC aggregates with Bmp for the initial 24 hr, followed by activin and Fgf for the next 2 days. The induced mesodermal cells were further posteriorized and maintained in the immature mesoderm state in the presence of a high concentration of Wnt agonist (CHIR 10  $\mu$ M) and Bmp, similar to mouse ESC induction. Given the physiological time period for caudal body extension in human embryos, we cultured EBs under these culture conditions for 6 days. Subsequently, we completely mimicked the protocol for mouse ESC differentiation by simply adjusting the culture periods (Figure 7A). The induced EBs harvested at day 14 expressed multiple signature genes for metanephric nephron progenitors (Figure 7B). Immunostaining revealed that many cells coexpressed typical nephrogenic transcription factors, including Wt1, Pax2, Sall1, and Six2 (Figure 7C). The induced EBs were further dissociated, and the induction efficiency was quantified by cytospin analyses. When the EBs were collected at day 11 (posterior intermediate mesoderm phase), more than 80% of the cells were positive for Wt1 (Figure S7A, *n* = 9). Analysis at day 14 (MM stage) showed that 20%–70% of the cells were positive for each representative metanephric nephron progenitor marker, including Wt1, Pax2, Sall1, and Six2 (Figure S7B, *n* = 9). Furthermore, these induced

progenitors exhibited robust tubulogenesis and clustered podocyte formation when cocultured with mouse embryonic spinal cords (Figures 7D and 7E, *n* = 6). Immunohistochemical examinations at day 8 revealed the formation of well-specified nephron components. These structures consisted of Wt1/nephrin<sup>+</sup> glomeruli (Figures 7F and 7G), cadherin6<sup>+</sup> proximal tubules (Figure 7H), and E-cadherin<sup>+</sup> distal tubules (Figure 7I), all of which appeared to be connected in that order, thereby mimicking human embryonic kidney formation. In conclusion, we succeeded in the induction of bona fide metanephric nephron progenitors and three-dimensional (3D) kidney structures from both mouse and human PSCs, by recapitulating the developmental processes *in vivo* (Figure 7J).

### DISCUSSION

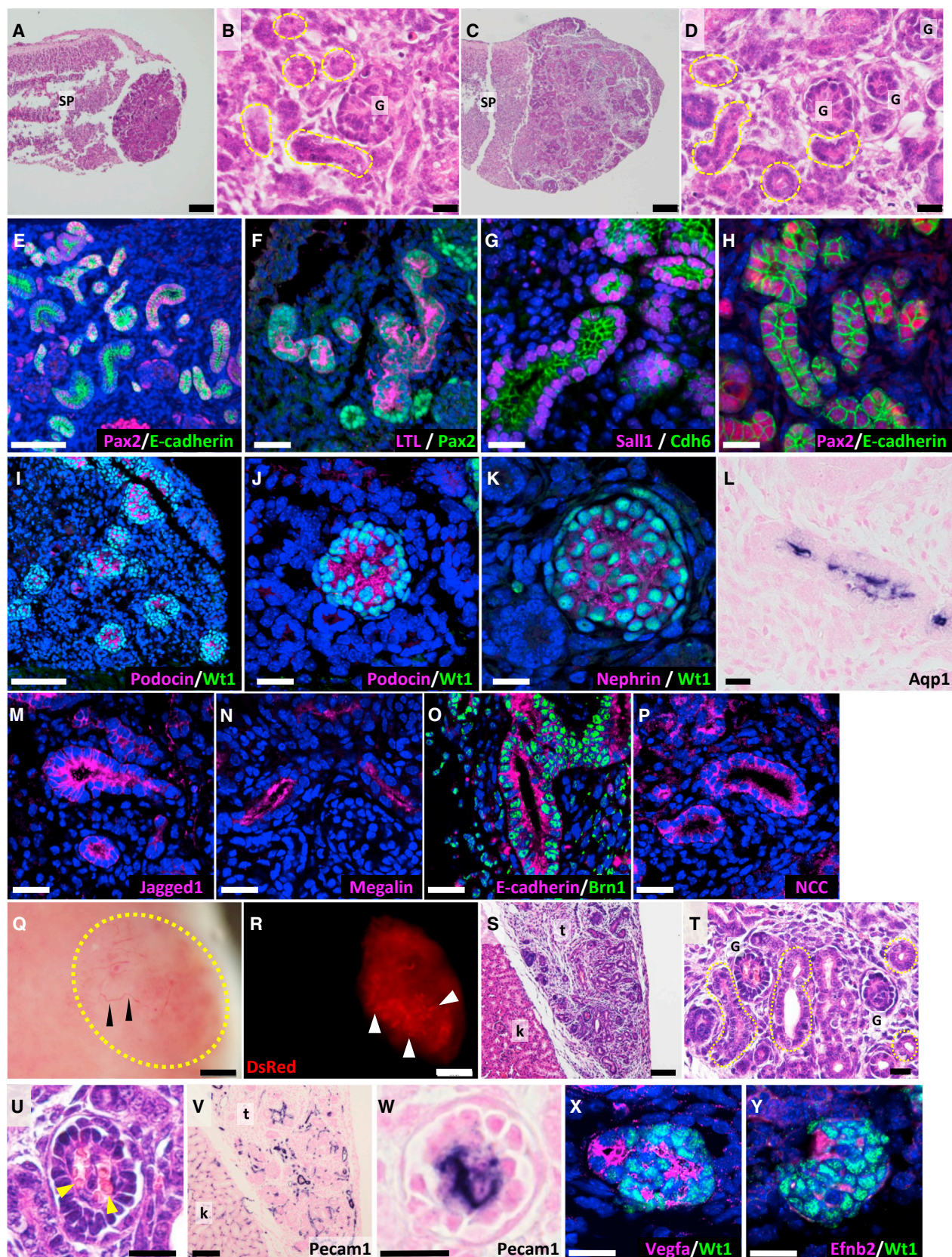
We have succeeded in inducing metanephric nephron progenitors capable of reconstituting 3D nephric tubules and glomeruli, the two main components that are critical for kidney functions, from mouse and human PSCs. These findings will be useful for revealing molecular mechanisms underlying human kidney diseases and open a way for regenerative medicine in the future. Our data also demonstrate that the developmental processes of the kidney are well conserved between humans and mice, and emphasize the robustness of our protocol that faithfully mimics the physiological processes. Our success relies on the approach that we initially determined the conditions for the later phase (from E9.5 to 10.5) and then progressed toward the earlier phases (from E8.5 to 10.5, and then ES to E10.5) by using embryonic tissue instead of starting from ESCs. The induction of kidney progenitors requires multiple steps, but reliable markers and detection assays were only available for the MM stage. Therefore, we could not have verified whether the induced ESCs in the early steps possessed the capacity to differentiate into nephron progenitors, unless all of the subsequent conditions had been optimized. Consequently, a conventional forward approach would not have been helpful. Our backward approach using fully “competent” embryonic precursors proved to be very effective and could be applicable to other organ induction.

*Brachyury* (T), which starts to be expressed at the gastrulation stage, has been recognized as a “transient” mesodermal marker in studies of directed differentiation from stem cells. However, it has recently been shown that the immature caudal population, namely the “axial progenitor,” persists in the posterior end of the embryo until the body axis extension is complete, and serves as the source of the caudal body trunk (Takemoto et al., 2011; Tzouanacou et al., 2009; Wilson et al., 2009). Our data suggest that this recently identified posterior precursor population could

### Figure 5. Metanephric Nephron Progenitor Induction from Mouse ESCs

- (A) Outline of the protocol is presented. A, activin; B, Bmp4; C, CHIR; R, retinoic acid; F, Fgf9.
- (B) Gene expression dynamics during the induction from ESCs is presented. The expression levels in the E10.5 embryonic MM are shown in red. The relative expression of each transcript to  $\beta$ -actin expression is presented as the mean  $\pm$  SEM (*n* = 4).
- (C) Localizations of Osr1-GFP/Wt1/Pax2<sup>+</sup> and Osr1-GFP/Sall1/Six2<sup>+</sup> cells in the induced EBs at day 8.5 are presented. The right two panels show higher-magnification images. Scale bars, 200  $\mu$ m (left eight panels) and 20  $\mu$ m (right two panels).
- (D) FACS analyses of Osr1-GFP and Itga8/Pdgfra expressions in EBs at day 8.5 are shown.
- (E) Gene expression analyses of the E11.5 Six2<sup>+</sup> embryonic nephron progenitor and stromal populations (Osr1<sup>+</sup>/Itga<sup>+</sup>/Pdgfra<sup>+</sup> and Osr1<sup>+</sup>/Itga<sup>low</sup>/Pdgfra<sup>+</sup>, respectively, sorted by FACS) and day 8.5 differentiated ESCs are shown. The relative expression of each transcript to  $\beta$ -actin expression is presented as the mean  $\pm$  SEM (*n* = 3).





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also be the origin of the MM, i.e., nephron progenitors, which argues against the conventional concept that the entire kidney is derived from the anteriorly formed intermediate mesoderm that extends caudally. The introduction of this axial progenitor model may be further applied for directed differentiation toward other caudally located organs.

In vitro, we succeeded in maintaining the T<sup>+</sup> state during the posteriorization phase by utilizing an unusually high concentration of Wnt agonist. Subsequently, graded attenuation of the Wnt agonist, as well as stage-specific addition of growth factors, allowed nephric lineage commitment and metanephric nephron progenitor formation. The necessity for high concentrations of the Wnt agonist in the initial phase of the induction could reflect the importance of Wnt signaling for the caudal body extension and maintenance of axial progenitors (Wilson et al., 2009; Yamaguchi et al., 1999). In the following step toward the posterior intermediate mesoderm, decreasing the Wnt agonist concentration and addition of retinoic acid are effective, similar to the caudal differentiation process of the paraxial mesoderm (Aulehla and Pourquié, 2010). The synergistic effect of activin on the nephric gene induction is reminiscent of a previous finding that a combination of activin and retinoic acid induces the pronephros-like structures in *Xenopus* animal caps (Moriya et al., 1993). Subsequently, addition of Fgf9 and a further reduction in Wnt signaling lead to the appearance of the metanephric nephron progenitors, and exogenous Bmp4, retinoic acid, and activin should be removed at this final step, indicating stage-specific requirements for each type of signaling (Figure 7J). These observations may partly explain why we failed to induce metanephric progenitors from Osr1<sup>+</sup> cells at E8.5. It is possible that Osr1<sup>+</sup> cells already present at E8.5 do not contribute to the metanephros and that the T<sup>+</sup>/Osr1<sup>−</sup> population gives rise to genuine T<sup>−</sup>/Osr1<sup>+</sup> cells that are competent for metanephros differentiation. The Osr1<sup>+</sup> cells that emerge during E8.5–E9.5 may have become labeled and contributed to the metanephros in a previously reported lineage-tracing study by Mugford et al. (2008b), although more detailed analyses are needed to test this possibility.

The nephron progenitors obtained from embryos readily form the 3D glomeruli and renal tubules when combined with spinal cord or upon Wnt stimulation. This should hold true for the progenitors induced in vitro, and our cells completely meet these criteria. Although a few studies have challenged the induction

of kidney progenitors (Mae et al., 2013; Narayanan et al., 2013), they lacked the ideas that the competence of intermediate mesoderm is temporally (E8.5 versus E9.5) and spatially (antero-posteriorly) different and considered Osr1<sup>+</sup> or Pax2<sup>+</sup> cells to be a homogeneous population. This could result in failure of the formation of 3D glomeruli, an essential feature of the metanephros. Thus, the capacity of the induced cells in the other reports would be quite different from that of our cells, despite the Osr1 or Pax2 expression.

Although we still need to further mature the nephron components, and also combine them with ureteric bud-derived structures to confer the physiological functions of the kidney, our study will be a big step toward kidney reconstruction in vitro. Combinations of developmental biology and stem cell biology, as shown in this report, will be effective to achieve this goal.

## EXPERIMENTAL PROCEDURES

### Sorted Embryonic Cell Culture

For embryonic tissue cultures at E8.5, the presomitic regions of six to ten somite-stage embryos were harvested, and T-GFP<sup>+</sup> cells were sorted by a FACSARIA II (Becton Dickinson). The sorted cells were aggregated at 7,000 cells per aggregate in 96-well low-cell-binding plates and cultured in serum-free chemically defined medium. In the embryonic tissue cultures at E9.5, the posterior regions from the 23<sup>rd</sup> somite region of 22–26 somite-stage embryos were harvested, and Osr1-GFP<sup>+</sup> or Wt1-GFP<sup>+</sup> cells were sorted. The sorted cells were aggregated at 10,000 cells per aggregate and cultured. All animal experiments were approved by the Animal Care and Use Committee of Kumamoto University (#A25-062).

### Mouse ESC and Human iPSC Culture

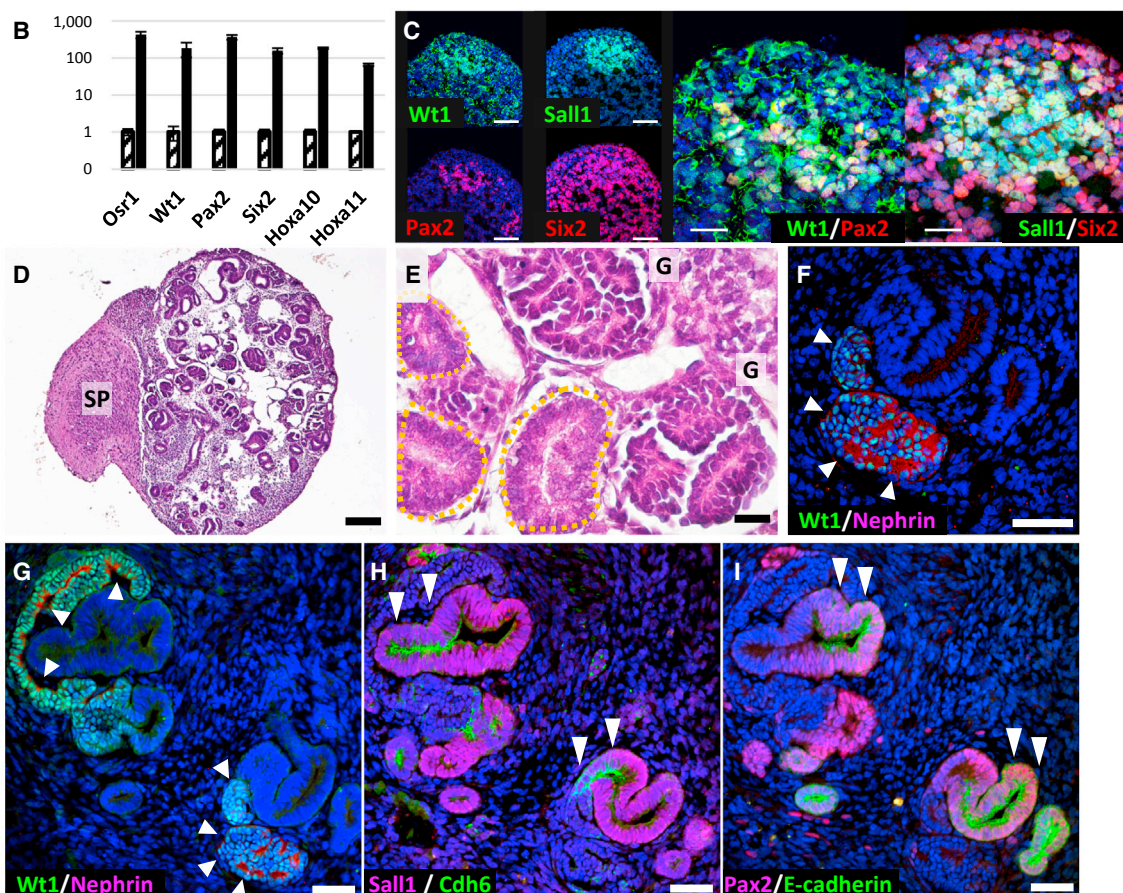
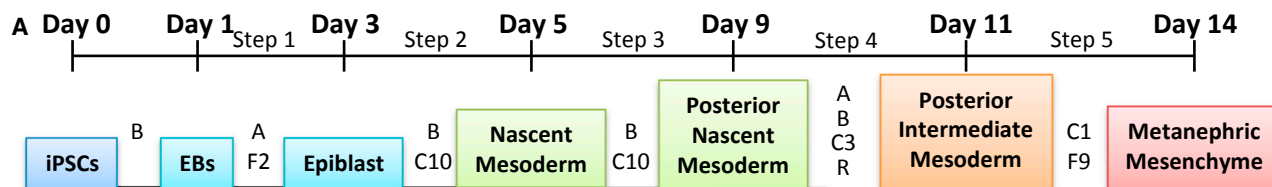
Mouse ESCs were aggregated at 1,000 cells per aggregate in 96-well U-bottom low-cell-binding plates to form EBs. After 48 hr (on day 2), the EBs were dissociated with Accutase and reaggregated in serum-free differentiation medium with addition of 0.5 or 1 ng/ml human activin A (R&D Systems). After 24 hr, the medium was switched to BC10 medium containing 1 ng/ml human Bmp4 (R&D Systems) and 10  $\mu$ M CHIR. After 36 hr, (on day 4.5), the medium was refreshed with new medium (BC10). On day 5.5, the medium was changed to ABC3R medium containing 10 ng/ml activin, 3 ng/ml Bmp4, 3  $\mu$ M CHIR, 0.1  $\mu$ M retinoic acid, and 10  $\mu$ M Y27632 (Wako). On day 6.5, the medium was changed to C1F medium containing 1  $\mu$ M CHIR, 5 ng/ml human Fgf9 (R&D Systems), and 10  $\mu$ M Y27632 (Wako).

Human iPSCs (201B7, Takahashi et al., 2007), which were obtained from RIKEN BioResource Center, were aggregated at 10,000 cells per aggregate in V-bottom 96-well low-cell-binding plates to form EBs, in the presence of 10  $\mu$ M Y27632 (Wako) and 0.5 ng/ml human Bmp4 (R&D Systems). After 24 hr (on day 1), the medium was changed to mesoderm-inducing medium

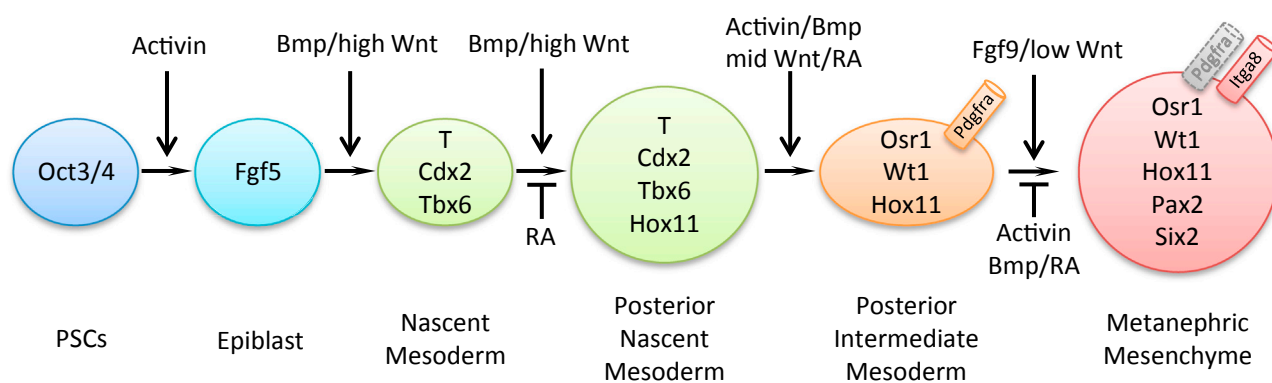
## Figure 6. 3D Kidney Structures from Mouse ESCs

(A–D) Hematoxylin and eosin staining shows the embryonic MM (A and B) and induced EB (C and D) cocultured with the spinal cord (higher magnification in B and D). The nephric tubules are outlined by dashed lines. SP, spinal cord; G, glomerulus. Scale bars, 200  $\mu$ m (A and C) and 20  $\mu$ m (B and D). (E–P) Immunostaining shows induced EBs for nephric tubule markers (E–H and L–P) and glomerular markers (I–K). Scale bars, 200  $\mu$ m (E), 100  $\mu$ m (I), and 20  $\mu$ m (F–H and J–P). (Q and R) Views present mouse kidneys excised at 1 week after being grafted with the induced EBs. The transplanted tissue is outlined by a dashed line. (Q) The black arrowheads show vascularization of the graft. (R) Fluorescence image shows the graft. The white arrowheads show nephron-like structures in the graft. Scale bars, 500  $\mu$ m. (S–U) Hematoxylin and eosin staining shows the grafted kidney sections. (S) Lower-magnification image is presented. t, transplanted tissue; k, host kidney. Scale bar, 100  $\mu$ m. (T) Medium-magnification image is shown. The nephric tubules are outlined by dashed lines. G, glomerulus. Scale bar, 20  $\mu$ m. (U) High-magnification image shows a glomerulus in the graft. The arrowheads indicate red blood cells in the vasculature invading the glomerulus. Scale bar, 20  $\mu$ m. (V and W) Immunostaining with blood vessel marker Pecam1 is shown. Scale bars, 20  $\mu$ m. (V) Lower-magnification image is shown. t, transplanted tissue; k, host kidney. (W) High-magnification image shows a glomerulus in the graft. (X and Y) Immunostaining shows glomeruli in the graft tissue for vascular growth factor markers. Scale bars, 20  $\mu$ m. See also Figure S6.





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containing 1 ng/ml human activin A and 20 ng/ml human basic Fgf (R&D Systems). After 48 hr (on day 3), the medium was switched to BC10 medium. Subsequently, half of the culture medium volume was refreshed with new medium every other day (BC10). On day 9, the medium was changed to ABC3R medium. On day 11, the medium was changed to C1F medium. All data shown are representative examples of at least three independent experiments, unless indicated otherwise.

#### Organ Culture of MM or Induced Metanephric Progenitors

The mesenchyme rudiments or induced ESC aggregates were cultured with embryonic spinal cord taken from E11.5 or E12.5 embryos or on 3T3Wnt4 cells at the air-fluid interface on a polycarbonate filter (0.8  $\mu$ m; Whatman) supplied with DMEM containing 10% fetal calf serum, as described previously by Kis-pert et al. (1998) and Osafune et al. (2006).

#### ACCESSION NUMBERS

The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GSE51054).

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2013.11.010>.

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#### Figure 7. Metanephric Nephron Progenitor Induction from Human iPSCs

- (A) Outline of the protocol is presented. A, Activin; B, Bmp4; C, CHIR; R, retinoic acid; F2, Fgf2; F9, Fgf9.
- (B) Gene expression analyses of the differentiated iPSCs are shown. The relative expression of each transcript to immature iPSCs (each left column) is presented as the mean  $\pm$  SEM (n = 6).
- (C) Localizations of Wt1/Pax2<sup>+</sup> and Sal1/Six2<sup>+</sup> cells in the induced EBs at day 14 are shown. The right two panels show higher-magnification images. Scale bars, 75  $\mu$ m (left four panels) and 20  $\mu$ m (right two panels).
- (D and E) Hematoxylin and eosin staining shows the induced EBs cocultured with spinal cord (D) and a higher-magnification image (E). The nephric tubules are outlined by dashed lines. SP, spinal cord; G, glomerulus. Scale bars, 200  $\mu$ m (D) and 20  $\mu$ m (E).
- (F–I) Immunostaining shows neighboring sections of induced EBs for glomerular markers (F and G) and nephric tubule markers (H and I). Arrowheads indicate the glomeruli in (F and G), the proximal tubule segments in (H), and the distal tubule segments in (I). Scale bars, 50  $\mu$ m.
- (J) Model of directed differentiation of PSCs toward the MM is presented.
- See also Figure S7.

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